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Use of Fungal Derived Polysaccharide-Conjugated Particles to Probe Dectin-1 Responses in Innate Immunity

Jenny M. Tam^{‡,a}, Michael K. Mansour^{‡,a}, Nida S. Khan^a, Nicholas C. Yoder^b, and Jatin M. Vyas^a

^a Department of Medicine, Division of Infectious Diseases, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, GRJ – 5 – 504, Boston, MA, 02114, USA.

^b Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142, USA Address, Address, Town, Country.

Abstract

The number of life-threatening fungal infections has risen in immunocompromised patients, and identification of the rules that govern an appropriate immune response is essential to develop better diagnostics and targeted therapeutics. The outer cell wall component on pathogenic fungi consists of β -1,3-glucan, and Dectin-1, a pattern recognition receptor present on the cell surface of innate immune cells, binds specifically to this carbohydrate. A barrier in understanding the exact immunological response to pathogen-derived carbohydrate epitopes is the presence of multiple types of carbohydrate moieties on fungal cell walls. To dissect the immunological mechanisms used to recognize pathogens, a system of “fungal like particles” was developed that consisted of polystyrene beads, which mimicked the three dimensional shape of the fungus, coated covalently with purified β -1,3-glucan derived from *Saccharomyces cerevisiae*. The morphology of the β -1,3-glucan layer was examined by immunofluorescence, flow cytometry, and immuno-transmission electron microscopy. The covalent linkages of the β -1,3-glucan to the polystyrene surface were stable after subjecting the beads to detergents. By pre-treating β -1,3-glucan beads with laminarinase, a specific β -1,3-gluconase, the reactivity of the anti- β -1,3-glucan antibody was abrogated in comparison to treatment with proteinase K indicating that the coating of these beads was predominantly β -1,3-glucan. TNF- α was also measured by stimulating bone-marrow derived macrophages with the β -1,3-glucan beads, and showed a dose dependent response compared to soluble β -glucan, insoluble β -1,3-glucan, uncoated beads, and soluble β -1,3-glucan mixed with uncoated beads. Finally, β -1,3-glucan beads were incubated with GFP-Dectin-1 expressing macrophages and imaged using confocal microscopy. β -1,3-beads were taken up within minutes and retained Dectin-1 recruitment to the phagosome as compared to uncoated beads. This data describes a unique fungal-like particle system that will permit immunologists to probe the critical steps in early recognition of pathogen-derived fungal carbohydrate antigens by innate immune cells.

Introduction

Invasive fungal infections, such as candidiasis, have become a leading cause of death among immunocompromised patients, *e.g.* leukemic patients, cancer patients with neutropenia and recipients of immunomodulatory agents including TNF- α blockers¹. In the intensive care

unit, candidal infections have become the third most common bloodstream infection after *Staphylococcus aureus* and *Pseudomonas aeruginosa*² and has a crude mortality rate of 30-40%². This high incidence highlights the need for better diagnostics and improved antifungal therapies. To improve outcomes for patients with invasive fungal infections, knowledge of the key molecular pathways within relevant immune cells, including a detailed understanding of the dynamic host-pathogen interactions upon phagocytosis, is mandatory in order to generate strategies to augment the host response.

Anti-fungal immunity is complex and involves both innate and adaptive immunity. Early innate defense includes phagocytes, such as macrophages, neutrophils, and dendritic cells, that recognize, ingest, and degrade the invading pathogen. Pattern-recognition receptors (PRRs) recognize highly conserved microbial epitopes known as pathogen-associated molecular patterns (PAMPs). The best-studied family of PRRs is the Toll-like receptors (TLRs). TLRs can provide strong activating signals to professional antigen presenting cells through activation of the NF- κ B pathway.³ Another PRR on the cell surface is Dectin-1, a type II membrane protein and C-type lectin that is highly expressed on phagocytes⁴. Dectin-1 recognizes the carbohydrate epitope β -1,3-glucan, which is a major PAMP⁵ and constitutes the major cell wall component of fungi such as *Candida albicans*, *Saccharomyces cerevisiae*, and *Aspergillus fumigatus*, though some β -1,6-linked side chains of varying length and distribution are also observed⁶⁷. Dectin-1 is required for proper modulation of immune responses, and reports have shown that patients with mutations in Dectin-1 are at higher risk for invasive fungal infections⁸⁹. The cytoplasmic tail of Dectin-1 contains an immunoreceptor tyrosine-based activation (ITAM)-like motif⁸. Upon ligation of the extracellular domain of Dectin-1, the tyrosine residue within the cytoplasmic ITAM motif is phosphorylated⁹. This phosphorylation event results in recruitment of spleen tyrosine kinase (Syk) and caspase recruitment domain protein 9 (Card9)¹⁰. Ultimately, this pathway leads to the activation of NF- κ B, and production of reactive oxygen species as well as pro-inflammatory cytokines including TNF- α and IL-12¹¹.

Isolated β -1,3-glucans can trigger immune responses, and there has been significant interest in their development as adjuvants and as immunotherapeutic agents¹². The ability of β -1,3-glucans to modulate immunity is influenced by their polymer length, degree of branching, and tertiary structure¹³. Although not yet fully understood, these attributes can influence the interaction of carbohydrate antigens with their cognate receptors, particularly Dectin-1¹⁴. Both soluble and insoluble β -1,3-glucan polymers have been shown to bind to Dectin-1, although only insoluble β -1,3-glucan activates Dectin-1 signaling to produce cytokine responses¹⁵. Zymosan, a derivative of the *S. cerevisiae* cell wall, has been used as a model substrate to define the immune response to fungal cell walls¹⁶. Zymosan particles are heterogeneously sized and composed of a complex mixture of polysaccharides and proteins whose exact composition is unknown. Thus, to characterize the Dectin-1 response to β -1,3-glucan antigens, we have constructed an “fungal-like particle” using purified β -1,3-glucan isolated from *Saccharomyces cerevisiae* covalently conjugated to a polystyrene bead platform. This platform allows direct probing of the Dectin-1 response to a pure β -1,3-glucan fraction, as opposed to the use of live organisms or cell wall particulates, which are complex in composition and contain multiple cell wall carbohydrate and protein components, all of which can generate non-Dectin-1-specific responses. The use of inert polystyrene beads as scaffold also replicates the size and geometry of the pathogenic yeast without the additional contribution of secreted products by the pathogen¹⁷ in addition to creating a monodisperse population to probe the immune response. A common approach cited in the literature is the use of physically adsorbed β -1,3-glucan to a polymeric bead surface^{17, 20}, but this approach fails to provide stability of the glucan layer on the bead surface, especially in the harsh degradative environments found in the endocytic pathway after phagocytosis.

Carbohydrates, including purified dextran, soluble β -1,3-glucan, and other synthetic polysaccharides have been immobilized using diverse chemical strategies including photochemical immobilization¹⁸, reductive amination¹⁹, EDC conjugation using carboxylate modified sugars²⁰, and utilizing divinyl sulfone (DVS)-modified surfaces²¹. These approaches require considerable synthetic chemistry expertise, either to install specific chemical functions on the carbohydrate of interest, or to construct the desired polysaccharide, or both. To probe the immune response to β -1,3-glucan by Dectin-1, we developed a simple method to attach purified carbohydrates from biologically relevant fungi to polymeric beads using minimal steps. The chemistry is based on activation of amine-functionalized beads with 1,1'-carbonyldiimidazole (CDI) and addition of soluble β -1,3-glucan to create a surface which mimics the yeast cell wall. We refer to these particles, which resemble pathogenic yeast in size and surface chemistry, as "artificial fungal pathogens".

These fungal-like particles were characterized using differential interference contrast (DIC) microscopy, immunofluorescence, and transmission-electron microscopy (TEM). The covalent interaction of the β -1,3-glucan layer to the surface of the bead was confirmed by a series of increasingly stringent detergent treatments. Purity of the β -1,3-glucan layer was also determined by incubating the beads with laminarinase, a specific β -1,3-gluconase. The amount of TNF- α produced by stimulating bone marrow derived macrophages was also measured by ELISA, and showed a dose dependent response when compared to soluble β -1,3-glucan, uncoated beads, and soluble β -1,3-glucan mixed with uncoated beads. Finally, Dectin-1 response was investigated by observing direct phagocytosis of β -glucan coated fungal-like particles by live RAW macrophages expressing a GFP-Dectin-1 fusion protein using spinning disk confocal microscopy. By providing a platform to probe directly immune responses to β -1,3-glucan, immunologists will now be able to dissect the critical steps in the early recognition of pathogen-derived fungal carbohydrate antigens in innate immunity. Additionally, this system permits the addition of other relevant pathogen-derived carbohydrates to polymeric beads to analyze the specific contribution of these ligands in the immune response.

Results and Discussion

β -1,3-glucan carbohydrates are covalently conjugated to polystyrene beads

1,1'-Carbonyldiimidazole (CDI) was used to conjugate amine-terminated polystyrene beads by activating the amino groups on the bead surface (Scheme 1). The resulting imidazole carbamate intermediates are sufficiently reactive to acylate primary and secondary hydroxyl groups without the use of strong bases. CDI has previously been used to conjugate hydroxyl-containing small molecules onto agarose beads, and the resulting carbamate linkage formed between the hydroxyl group and the bead surface is noncharged and highly stable under immunoprecipitation conditions²². Therefore, this strategy was used to conjugate β -1,3-glucan to amino-terminated microspheres. Following CDI activation, a DMSO solution of β -1,3-glucan was incubated with the beads to complete the coupling.

The amount of β -1,3-glucan on the surface of the bead was analyzed using a phenol-sulphuric acid assay²³. The average micrograms of β -1,3-glucan on the surface of the bead was 7.54×10^{-8} μ g. The amount of β -1,3-glucan on the bead surface, as analyzed by the phenol-sulphuric acid assay, correlated to the amount of β -1,3-glucan seen by flow cytometry (data not shown).

Preparations of β -1,3-glucan that are insoluble in water elicit stronger innate immune responses than soluble preparations, likely due the higher valency of the insoluble material endowing an increased capacity to crosslink Dectin-1 at the cell surface¹⁵. We therefore

utilized soluble β -1,3-glucan from *S. cerevisiae*, which we observed also to be soluble in DMSO. The use of DMSO has the additional advantage of minimizing any competing hydrolysis of surface imidazole carbamates during the reaction. Coupling of β -1,3-glucan is expected to proceed mostly through the primary hydroxyl groups of the polysaccharide chain, although a small fraction of coupling through the secondary hydroxyl groups is also expected²².

Our ability to label β -1,3-glucan beads with NHS ester dye after carbohydrate conjugation strongly suggests that additional amine sites not activated by CDI are present on the bead surface. These sites would be available for conjugation with other dyes or with other biomolecules such as pathogen-derived proteins or peptides. By incorporating separate cell wall components to the polymer bead, distinct host responses to combinations of individual pathogen components could be ascertained.

Beads characterized by immunofluorescence, flow cytometry, and transmission electron microscopy (TEM) show uniform coating of β -1,3-glucan to the microsphere surface

The carbohydrate layer on the coated beads was examined using differential interference contrast (DIC) microscopy to examine gross surface morphology of the beads and confocal immunofluorescent microscopy using an antibody against β -1,3-glucan (Fig. 1). By DIC, both the β -1,3-glucan-coupled and uncoated beads show the same circular structure (Fig. 1A, 1D). After incubation with anti- β -1,3-glucan, the β -1,3-glucan coated beads exhibit strong fluorescence, as compared to the uncoated bead. To determine any non-specific binding, both beads were also incubated with an isotype control antibody and neither bead type exhibited detectable fluorescence (data not shown). Flow cytometry was also used to compare the distribution of carbohydrate on the labeled beads (Fig. 1B, 1E). The beads coated in β -1,3-glucan show a sharp peak, indicated in red, showing that the β -1,3-glucan beads are uniformly and densely coated specifically with the glucan (Fig. 1B). Incubation of the β -1,3-glucan coated bead with the isotype control antibody showed little to no fluorescence signal (data not shown). In contrast, the peak from the uncoated beads coated with the anti- β -1,3-glucan overlaps almost exactly to that of the isotype control antibody (Fig. 1E).

Immuno-TEM was also performed with the β -1,3-glucan beads to examine the surface of the beads as well as confirm additional antibody specificity to the β -1,3-glucan surface (Fig. 1C, 1F). The surface of the β -glucan-coated beads shows a diffuse layer of electron density <50 nm in thickness not present on unmodified beads, which we presume is indicative of the carbohydrate layer. The anti- β -1,3-glucan gold nanoparticles observed on the bead surface (Fig. 1C) show that this surface feature is specifically β -1,3-glucan. In contrast, the surface of the uncoated beads (Fig. 1F), show a smooth surface with no gold particles bound to the surface.

β -1,3-glucan layer is stable with incubated with detergents

The stability of the carbamate linkage between the carbohydrate and the polystyrene beads was examined by subjecting the coupled β -1,3-glucan beads to detergents in concentrations typically used in immunoprecipitation: 3% SDS, 1% NP40, and 1% Brij 58, and analyzed by flow cytometry (Fig. 2A). After incubating the β -1,3-glucan coated beads with each detergent followed by rinsing, the beads were incubated with the β -1,3-glucan antibody to determine the stability of the β -glucan coat and assessed by flow cytometry. Surface staining of β -1,3-glucan was unchanged despite the detergent exposures. As a positive control, the β -1,3-glucan coated beads were also incubated in PBS prior to antibody labeling.

β -1,3-glucan layer can be selectively digested using laminarinase

We also wanted to examine effect of different gluconases to assess further the specificity of the carbohydrate layer on the bead. A specific β -1,3-gluconohydrolase, laminarinase, was used (Fig. 2B) and compared to the control protease, proteinase K (Fig. 2C). β -1,3-glucan beads were mixed with two concentrations of laminarinase, 0.5 U/mL and 0.1 U/mL, as well as in 100 mM sodium acetate buffer alone, as a control. Laminarinase hydrolyses oligomers of glucose from glucan chains²⁴. By analysis with flow cytometry, both concentrations of laminarinase removed the β -glucan layer, evidenced by their peaks close to that of beads incubated with isotype control antibody. In contrast, when exposed to proteinase K, the β -1,3-glucan coat remained intact on the bead surface, as shown in flow cytometry (Fig. 2C). Proteinase K is a broad-spectrum serine protease and was used at two concentrations, 127 U/mL and 25 U/mL. Neither concentration showed alteration of the β -1,3-glucan layer as measured by flow cytometry using β -1,3-glucan antibody. This observation is consistent with immobilization of β -1,3-glucan through the saccharide hydroxyl groups and not through any protein contaminant present in the cell wall preparation.

β -1,3-glucan beads induce TNF- α responses in a dose dependent manner

To determine if β -1,3-glucan beads were competent to trigger an inflammatory response in professional antigen presenting cells, we incubated the β -1,3-glucan beads with immortalized bone marrow-derived macrophages (BMDM). As expected, the β -1,3-beads induced a higher TNF- α response when compared to soluble β -1,3-glucan alone, uncoated beads, and uncoated beads mixed with β -1,3-glucan as analyzed through ELISA. Since native BMDM does not exhibit appreciable amounts of Dectin-1 expression as determined by Western blotting (data not shown), these cells were transduced with GFPDectin-1 in order to measure TNF- α response. The soluble β -1,3-glucan used to stimulate the BMDM was the same carbohydrate used to create the β -1,3-glucan beads.

After 24 hrs of stimulation of the BMDM with the aforementioned ligands, the β -1,3-glucan beads showed a significantly higher TNF- α response compared to soluble β -1,3-glucan mixed with uncoated beads, uncoated beads, and soluble β -1,3-glucan alone (Fig. 3). These results indicate that the carbohydrate must be bound to the surface of a particle in order to induce a TNF- α response. In addition, as the effector to target ration (E:T) is increased, the TNF- α response increases accordingly. The TNF- α response of the β -1,3-glucan beads is comparable to stimulation of BMDM with 2 μ g/mL of insoluble β -1,3-glucan.

Dectin-1 is specifically recruited to phagosomes containing β -1,3-glucan beads

Dectin-1 is the major β -1,3-glucan receptor expressed on the surface of immune cells including macrophages and dendritic cells²⁵. We probed the immunological response of RAW 264.7 (RAW) macrophages expressing GFP-Dectin-1 using our β -1,3-glucan coupled beads (Fig. 4, column A) and compared the response to live *C. albicans* (Fig. 4, column B), and live *S. cerevisiae* (Fig. 4, column C), and uncoated polystyrene beads (Fig. 4, column D). When the β -1,3-glucan coated beads are phagocytosed by the RAW macrophages, GFP-Dectin-1, on the cell membrane, is immediately recruited to the phagosome, indicated by a bright green ring (Fig. 4, column A). The fluorescence from GFP-Dectin-1 surrounding the fungal β -1,3-glucan bead has similar intensity to GFP-Dectin-1 surrounding *C. albicans* (Fig. 4, column B) and *S. cerevisiae* (Fig. 4, column C), indicating that the β -1,3-glucan beads elicit Dectin-1 responses similar to live fungi. The fluorescent signal from GFP-Dectin-1 and the β -1,3-glucan coated bead co-localize in the merged image (Fig. 4, column A). In comparison, when RAW cells expressing GFP-Dectin-1 ingest uncoated polystyrene beads (Fig. 4, column D) there is no GFP-Dectin-1 recruitment to the phagosome, indicating that Dectin-1 recruitment is specific to β -1,3-glucan and not the bead scaffold.

Experimental

Preparation of β -1,3-glucan conjugated microspheres

300 μ L of stock solution containing 3 μ m amine-coated polystyrene beads (1.67×10^9 beads/mL, Polysciences, Warrington, PA) were washed three times in dimethylsulfoxide (DMSO) (Sigma-Aldrich) using centrifugal filters containing a 0.45 μ m PTFE membrane (Ultrafree brand, Millipore, Billerica, MA). Beads were resuspended in anhydrous DMSO. The amino groups on the beads were then activated by adding 1,1'-carbonyldiimidazole (CDI, Sigma-Aldrich) at a final concentration of 0.5 M CDI in anhydrous DMSO. The bead/CDI mixture was then shaken at room temperature for 1 hour. Beads were rinsed three times in anhydrous DMSO using centrifugal filters, then resuspended in DMSO. Approximately 10 mM of β -1,3-glucan (Wellmune, Biothera, *Saccharomyces cerevisiae*, Eagan, MN) dissolved in DMSO was added. The mixture was shaken at room temperature for 1 hour. The beads were filtered using centrifugal filters to remove DMSO, and resuspended in PBS. After washing 3X in PBS, the bead mixture was resuspended in PBS and stored at 4°C. Carbohydrate content on the bead surface was analyzed using the phenol-sulfuric acid assay ²³.

After carbohydrate loading, beads were also fluorescently dyed for live-cell imaging by mixing an aliquot of beads ($\sim 50 \times 10^6$) with 30 μ g of N-hydroxysuccinimidyl ester activated Alexa Fluor (AF) dye (Invitrogen) for 1 hour at room temperature. For labeling experiments, AF405 (ex: 401/em: 421) and AF647 (ex: 650/em: 655) were used. Excess dye was removed with 3X washes in PBS and the beads were immediately used in imaging experiments.

Immuno-Transmission Electron Microscopy (TEM) OF β -1,3-glucan microspheres

The primary antibody mouse-anti- β -1,3-glucan (Biosupplies, Parksville, Australia) was incubated for 1 hour at RT with an equal volume of secondary goat-anti-rabbit labeled labeled 15 nm gold (Ted Pella, Redding, CA) at final concentrations of 1:10 for the anti- β -1,3-glucan and 1:30 for the secondary antibody. This antibody-gold mixture was then pelleted and incubated with the bead samples for one hour at room temperature. Beads were pelleted again and rinsed once in 0.1 M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences (EMS), Hatfield, PA). They were then post-stained with a mixture of 0.1% ruthenium red (EMS) plus 1.0% osmium tetroxide (EMS) in cacodylate buffer for one hour at room temperature. They were pelleted again and rinsed once with buffer, then stabilized in 2.0% agarose for ease of handling. The pellets were dehydrated through a graded series of ethanol washes and embedded in Eponate resin (Ted Pella, Redding, CA) at 60°C overnight. Thin sections were cut on a Leica EM UC7 and collected onto formvar-coated slot grids. Sections were examined in a JEOL 1011 transmission electron microscope at 80 kV and images were collected using an AMT digital imaging system (Advanced Microscopy Techniques, Danvers, MA).

Analysis of β -1,3-glucan microspheres by flow cytometry

Approximately 50,000 beads, as determined by manual counting using a hemacytometer, were incubated in 400 μ L of PBS with 2% bovine serum albumin (BSA, Sigma Aldrich) (PBS/BSA) for 1 hour at room temperature. After washing, 0.25 μ g of anti- β -1,3-glucan antibody (monoclonal mouse IgG, Biosupplies, Parksville, Australia) or irrelevant isotype control (anti-AP3, mouse IgG1, Santa Cruz Biotechnology, Santa Cruz, CA) was added for 1 hour at room temperature in PBS/BSA solution and then washed 3X in PBS/BSA solution. Beads were resuspended in 400 μ L of PBS/BSA and 2 μ g of polyclonal rabbit anti-mouse IgG-Alexa Fluor 488 antibody (Molecular Probes, Eugene, OR) was added for 1 hour at room temperature in the dark. After washing, beads were resuspended in 400 μ L PBS/BSA and assessed for surface fluorescence on a FACS Calibur (Becton-Dickinson, Franklin

Lakes, NJ). Flow cytometry analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR).

Stability of β -1,3-glucan when exposed to detergents

Approximately 50,000 β -1,3-glucan microspheres were incubated with various detergent conditions oscillating for 1 hour at 4°C. SDS (1% and 0.5%) (Sigma-Aldrich), NP-40 (1%) (Sigma-Aldrich), and Brij (1% Brij 58, 10 mM Tris [pH 7.8], 150 mM NaCl, 5 mM MgCl₂, 0.5%) (Sigma-Aldrich) detergents were used to test the stability of the β -1,3-glucan layer on the bead surface. After washing with PBS/BSA, β -1,3-glucan microspheres were assessed for β -1,3-glucan content using immunofluorescence by flow cytometry as described above.

Digestion of β -1,3-glucan with laminarinase

Approximately 25,000 β -1,3-glucan beads were placed into 0.1 M sodium acetate buffer, pH 5.0 (Sigma-Aldrich) or PBS. Several concentrations of laminarinase (derived from *Trichoderma* species, Sigma-Aldrich) (0.1 U/mL and 0.5 U/mL) or proteinase K (Sigma-Aldrich) (25 U/mL and 127 U/mL) were added and incubated overnight at 37°C on a rotating drum. Beads were washed with PBS/BSA solution and then analyzed for β -glucan content using the phenol-sulfuric method, confocal microscopy or immunofluorescence as described.

RAW 264.7 cell culture

RAW 264.7 macrophage cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (HyClone, ThermoScientific, Logan, UT), 1% penicillin/streptomycin (Gibco, Invitrogen, Carlsbad, CA), and 1% L-glutamine (Gibco, Invitrogen). Cells were grown at 37°C with 5% CO₂ for two days to ensure log-phase growth of the cells in order to optimize their ability to engulf particles. Two hours prior to imaging, RAW cells were re-plated into the chambered coverglass with DMEM medium containing 20% FBS, 2% penicillin/streptomycin, and 2% L-glutamine.

Lentiviral transduction

HEK293T cells were cultured in DMEM containing 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin as described previously²⁶ and used to generate lentivirus. eGFP-Dectin-1 (kind gift from David Underhill) was subcloned into the pHAGE II vector, a fourth-generation lentiviral self-inactivating nonreplicative vector used for transduction of bone marrow stem cells²⁷. Lentivirus was made as described²⁶. Cellular transduction was done in 12-well plates whereby 100 μ L of concentrated viral supernatant was placed onto plated cells and incubated at 37°C overnight. Supernatants were replaced with normal media the following day, and cells were selected using 5 μ g/mL puromycin. Successful infection was determined by puromycin resistance and direct examination under an epifluorescence microscope for the presence of green fluorescence.

Measuring TNF- α production using ELISA

Immortalized bone marrow-derived macrophages (BMDM) from BL6 mice (kind gift from Dr. Douglas Golenbock) were transduced with GFP-Dectin-1 lentivirus and plated in a 96-well plate at 5×10^4 cells/well. Cells were stimulated with the following ligands and corresponding concentrations or effector to target cell ratios (E:T): soluble β -1,3-glucan (2 μ g/mL, 20 μ g/mL, 200 μ g/mL), β -1,3-glucan beads (E:T of 10:1, 20:1, and 30:1), uncoated beads (E:T of 10:1, 20:1, and 30:1), soluble β -1,3-glucan mixed with uncoated beads (2 μ g/mL, 20 μ g/mL, 200 μ g/mL – each concentration mixed with uncoated beads at an E:T of 20:1). Insoluble β -1,3-glucan (2 μ g/mL) was also used as a positive control. BMDM were stimulated for 24 hrs, and fresh supernatants were analyzed using an ELISA assay

measuring TNF- α (DuoSet, Dy410) developed by R&D Systems (Minneapolis, MN). Statistical analysis was done in GraphPad Prism4 using Two-Way ANOVA with Bonferroni post tests.

Confocal microscopy

GFP-Dectin-1 RAW cells, or β -1,3-glucan microspheres immunostained with anti- β -1,3-glucan antibody were plated onto confocal dishes (LabTek, ThermoScientific, Rochester, NY). Cells were incubated with AF647 dyed- β -1,3-glucan microspheres for 30 min at 37°C $\times$ 5% CO₂ in complete media (DMEM supplemented with 10% FBS, penicillin, streptomycin, and L-glutamine). After washing, slides were mounted onto a Nikon Ti-E inverted microscope fitted with a spinning disc confocal detection head (Yokogawa, Sugar Land, TX). A 4 W, continuous-wave laser (Coherent, Santa Clara, CA) or solid-state UV laser were used to produce excitation wavelengths of 405nm or 647nm. Cells and beads were imaged using a Nikon 100X objective with high numerical aperture (Nikon, 1.49 NA, oil immersion). Images were captured using an EM-CCD camera (Hamamatsu, C9100-13, Bridgewater, NJ) and analyzed using Metamorph software (Molecular Devices, Downingtown, PA)²⁸.

Conclusions

In summary, we have developed a convenient method to prepare β -1,3-glucan coated particles that serve as “fungal-like particles” and elicit a specific anti-fungal Dectin-1 response in mammalian immune cells. These beads engaged Dectin-1, as shown by confocal fluorescence microscopy, and we measured inflammatory cytokine responses, specifically TNF- α in bone marrow-derived macrophages. This system allowed specific analysis of TNF- α to a pure β -1,3-glucan fraction, as opposed to using cell wall particulates, which contain multiple cell wall carbohydrates and other protein components, generating non-specific responses to Dectin-1. Due to the stability and purity of the β -1,3-glucan surface, we expect that these beads will prove useful for immunoprecipitation studies to purify receptors and other proteins associated with this fungal-derived carbohydrate. Additional available amine sites on the bead surface also permit the incorporation of fluorescent labels to track beads during cell interactions. These additional conjugation handles may allow incorporation of other relevant pathogen-derived molecules to dissect the specific contribution of these ligands in the immune response. In addition this technology could be applied to beads consisting of different materials. For example, magnetic beads with an amine-coated surface could also be used to coat carbohydrates on the surface, which could then be subsequently used for isolation of phagocytic cells. Thus, these beads point the way toward multifunctional adjuvant or immunotherapeutic agents for modulating immune responses.

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Scheme 1.

Overview of the β -1,3-glucan conjugation scheme. Amine-terminated microspheres were incubated in a solution of 0.5 M 1,1'-carbonyldiimidazole in DMSO for 1 hour at room temperature. After washing, beads are added and shaken in a solution of β -1,3-glucan derived from *S. cerevisiae* dissolved in DMSO. β -1,3-glucan forms a linkage through the primary hydroxyl on the 6th position of the saccharide.

Figure 1. Surface characterization of β -1,3-glucan beads

(A) Immunofluorescence using anti- β -1,3-glucan antibody comparing β -1,3-glucan conjugated beads with (D) unconjugated beads under DIC and confocal microscopy. (B,E) Flow cytometry comparing (B) β -1,3-glucan beads compared with (E) unconjugated beads incubated with anti- β -1,3-glucan antibody conjugated to Alexa fluor 488. (C,F) Immunotransmission electron micrographs of (C) β -1,3-glucan beads to (F) uncoated beads; anti- β -1,3-glucan antibody visualized with gold nanoparticles (arrows).

Figure 2. β -1,3-glucans are conjugated to microspheres through covalent bonds
 (A) β -1,3-glucan beads were treated with increasingly stringent conditions for 1 hour. (B) β -1,3-glucan beads were treated with laminarinase, a specific β -1,3-gluconase, as compared to (C) proteinase K. In all conditions, surface β -1,3-glucan was detected using anti- β -1,3-glucan antibody.

Figure 3. β-1,3-glucan beads induce TNF-α secretion in a dose dependent manner

TNF-α production was assessed by ELISA, and show that the β-1,3-glucan beads induce a higher TNF-α response in immortalized bone marrow-derived macrophages as compared to soluble β-1,3-glucan, uncoated beads, and soluble β-1,3-glucan mixed with uncoated beads. The β-1,3-glucan beads give a comparable response to the positive control, insoluble β-1,3-glucan. Data are means plus standard deviation of triplicate culture (**p, < 0.001).

Figure 4. Dectin-1 recruitment to β -1,3-glucan beads as compared to *C. albicans* and *S. cerevisiae* in live macrophages

(column A) β -1,3-glucan beads (red) ingested by GFP-Dectin-1 expressing RAW macrophages as compared to (column B) *C. albicans*, (column C) *S. cerevisiae*, and (column D) unconjugated beads (blue). Incubation time is 30 min at 37°C. Bar indicates 5 μ m.